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Water influx and cell swelling after nanosecond electropermeabilization

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ABSTRACT

Pulsed electric fields are used to permeabilize cell membranes in biotechnology and the clinic. Although molecular and continuum models provide compelling representations of the mechanisms underlying this phenomenon, a clear structural link between the biomolecular transformations displayed in molecular dynamics (MD) simulations and the micro- and macroscale cellular responses observed in the laboratory has not been established. In this paper, plasma membrane electropermeabilization is characterized by exposing Jurkat T lymphoblasts to pulsed electric fields less than 10 ns long (including single pulse exposures), and by monitoring the resulting osmotically driven cell swelling as a function of pulse number and pulse repetition rate. In this way, we reduce the complexity of the experimental system and lay a foundation for gauging the correspondence between measured and simulated values for water and ion transport through electropermeabilized membranes. We find that a single 10 MV/m pulse of 5 ns duration produces measurable swelling of Jurkat T lymphoblasts in growth medium, and we estimate from the swelling kinetics the ion and water flux that follows the electropermeabilization of the membrane. From these observations we set boundaries on the net conductance of the permeabilized membrane, and we show how this is consistent with model predictions for the conductance and areal density of nanoelectropulse-induced lipid nanopores.

1. Introduction

By altering the barrier function of the cell membrane in a non-lethal manner, electropermeabilization (electroporation) facilitates the entry of nucleic acids and pharmaceutical agents into living cells [1,2]. Clinical applications of electroporation include electrochemotherapy (enhancement of anti-tumor drug activity) [3], electrogene therapy (promotion of the uptake of cancer-suppressing genetic material) [4], and non-thermal ablation of cancer tissue [5–7]. Efficient optimization of the protocols used in these applications requires an expansion of our knowledge of the mechanisms of the restructuring of cell membranes that occurs in the presence of an applied electric field.

Investigations following the first reports of modifications of membrane conductance by electric fields [8–10] led to an empirical understanding of the electropermeabilization process [11–13] that was complemented by electrophysical models [14–20], and more recently corroborated by molecular dynamics (MD) simulations [21–26]. Taken together, these studies have given us a hypothetical physical mechanism for the poration of phospholipid bilayers, which can be expected to apply, at least in part, to the electropermeabilization of living cell membranes. It must be kept in mind, however, that the MD systems studied to date are highly simplified in comparison with a cell membrane, with one or two or at most three lipid and one or two cation species in bilayers with areas less than 200 nm^2, containing no membrane proteins or other intra- and extracellular associations.

As the availability of increasing computing power enables us to simulate more and more complex systems (larger and more heterogeneous bilayers with embedded proteins and intra- and extracellular attachments), we seek also a simplification of our experimental systems, to approach in our observations the primary events of the electropermeabilization process, separating them as much as possible from the myriad downstream consequences of breaching the cell membrane barrier with a large external electric field. At the intersecting trajectories of more and more accurate models and more and more fundamental experiments, we expect to find not only a better understanding of the physics of single, isolated lipid nanopores but also a mechanistic framework for manipulating populations of electric field-generated pores in the cells and tissues of living systems [27].

In this paper we simplify the experimental system in two ways. First, we minimize the duration of the permeabilizing event to less than 10 ns [28–31], and we include in our analysis cell responses to single pulses. In this way we reduce the number and the magnitude of secondary

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disturbances. The rationale for this approach can be explained by considering that when applying a train of pulses, each new pulse acts on cells that have been already affected by the preceding exposure, triggering a series of cascading events that increase the complexity of the phenomena to be analyzed. Applying only a single pulse simplifies the experimental conditions and the cellular responses. Similarly for pulse duration, as the field is applied for a longer and longer time, it is reasonable to expect that the initial primary effects of field-biomolecular structure interaction will be compounded. Therefore, short pulse studies like this one can help to unravel the complexity and to approach the most basic events of the electropermeabilization process.

Second, rather than use impermeant dyes as indicators of electroporation, we track the changes in cell volume that occur as a result of membrane permeabilization-induced osmotic imbalance [32–36]. Electropermeabilized membranes are conductive for Na^+, K^+, and Cl^-, so a permeabilized cell cannot maintain the normal transmembrane concentration gradients for these ions, a key component of the net cellular osmotic balance. In physiological media, because larger intracellular solutes cannot cross the permeabilized membrane, the intracellular osmolality becomes greater than the extracellular osmolality. This is countered by water influx into the cell, resulting in an increase in cell volume (cell swelling) [37,38]. The advantage of this approach compared to methods based on fluorescent, impermeant dyes for the study of electropermeabilization induced by nanosecond pulses is that the sensitivity of the latter is limited by the number and size of the pores created. Below a certain dose, the fluorescence emission from the small amount of dye that enters the cell is difficult to distinguish from background with affordable and flexible imaging and other detection systems. It has been demonstrated for nanosecond pulses that the process of pore creation dominates that of pore expansion, so the transport of even relatively small dye molecules through the nanometer-sized pores produced by nanosecond pulse exposure is much less than the flux of smaller species like calcium and monovalent ions [39]. Thus the analysis of cell swelling, which is not dependent on the dye selection and on the sensitivity of fluorescence detection methods, can be used for the study of plasma membrane electropermeabilization even in response to mild stresses (single, nanosecond-duration pulses), and for following the time dynamics of the phenomenon [38].

By exposing Jurkat T lymphoblasts to 5 ns, 10 MV/m pulsed electric fields, we are able to systematically analyze, using standard white light microscopic imaging, the resulting osmotically driven cell swelling as a function of pulse number (from 1 to 50) and pulse repetition rate (1 kHz or 1 Hz). From the rate and magnitude of the response, we characterize the dynamics of electropermeabilization under relatively unexplored pulsing conditions, and we extract values for ion and water flux through the permeabilized membrane.

This approach provides a simple and direct connection between simulations and experimental systems. By correlating observed swelling kinetics with rates of pore formation and ion and water transport obtained from molecular simulations and continuum representations, we establish reference points for improving the accuracy and applicability of the models, we elevate confidence in the validity of the mechanism for electropermeabilization that is emerging from MD studies, and we strengthen the physics-grounded foundation needed for methodological improvements in electroporation technology.

2. Materials and methods

2.1. Cell experiments

2.1.1. Cell culture

Jurkat T lymphoblasts (ATCC TIB-152, Manassas, VA) were grown in RPMI 1640 (Mediatech, Manassas, VA) containing 10% heat-inactivated fetal bovine serum (Gibco, Carlsbad, CA), 2 mM L-glutamine (Gibco), 50 U/mL penicillin (Gibco), and 50 μg/mL streptomycin (Gibco). Cells were maintained in exponential growth at 37 °C in a humidified, 5% CO_2 atmosphere.

2.1.2. Cell preparation

For pulse treatment, cells were concentrated to 2×10^7 cells/mL and in some cases incubated for 15 min with 0.5 μM calcein-AM (acetoxymethyl ester, Molecular Probes, Eugene, OR), which enhances visualization of the cell outline using fluorescence microscopy. After loading of the dye, cells were centrifuged and resuspended in fresh RPMI 1640 (or in some cases 150 mM NaCl) at 2×10^7 cells/mL. For exposure in presence of lanthanide or mercuric ions, $GdCl_3$ or $LaCl_3$ (Aldrich Chem. Co, Milwaukee, WI), 100 μM or 1 mM, or $HgCl_2$, 50 μM, was added to the cell suspension 5 min before pulse treatment.

2.1.3. Pulsed electric field exposures

For microscopic observation, cells were placed in a microchamber 100 μm wide, 30 μm deep, and 15 mm long, with platinum electrode walls on a glass microscope slide. A resonant-charged, solid-state Marx bank-driven, hybrid-core compression, diode-opening switch pulse generator designed and assembled at the University of Southern California [40] delivered 5 ns, 10 MV/m electrical pulses at a 1 kHz repetition rate (1 Hz where noted) to the microchamber electrodes mounted on the microscope stage in ambient atmosphere at room temperature.

2.1.4. DIC and fluorescence microscopy

Observations of live cells during and after pulse exposure were made with a Zeiss (Göttingen, Germany) Axiovert 200 epifluorescence microscope with 63× water immersion objective and Hamamatsu (Higashi-ku, Hamamatsu City, Japan) ImageEM EM-CCD camera. Captured images were analyzed with Hamamatsu SimplePCI and ImageJ (http://imagej.nih.gov/ij/) software. The cell perimeter was tracked using a freehand selection function, and then the area defined by the drawn perimeter was measured. To reduce variability, cells in the center of the exposure chamber, not adjacent to the electrode surfaces, were selected in the images captured immediately before pulsing, and then the same cells were analyzed in the post-pulse images. For each pulsing condition, at least 3 experiments were carried out, and a total of at least 30 cells were analyzed.

2.2. Molecular dynamics simulations

2.2.1. Simulation conditions and parameters

Simulations were performed using the GROMACS set of programs version 4.0.5 [41] on the University of Southern California High Performance Computing and Communications Linux cluster (http://www.usc.edu/hpcc/). Lipid topologies derived from OPLS united-atom parameters [42] were obtained from Peter Tieleman (http://moose.bio.ucalgary.ca). The Simple Point Charge (SPC) water model [43] was used, and all simulations were coupled to a temperature bath at 310 K with a relaxation time of 0.1 ps and a pressure bath at 1 bar with a relaxation time of 1 ps, each using a weak coupling algorithm [44]. Pressure was coupled semi-isotropically (using a compressibility of 4.5×10^{-5} bar^{-1}) normal to and in the plane of the membrane (NpT). Bond lengths were constrained using the LINCS algorithm [45] for lipids and SETTLE [46] for water. Short-range electrostatic and Lennard–Jones interactions were cut off at 1.0 nm. Long-range electrostatics was calculated with the PME algorithm [47] using fast Fourier transforms and conductive boundary conditions. Reciprocal-space interactions were evaluated on a 0.12 nm grid with fourth order B-spline interpolation. The parameter ewald_rtol, which controls the relative error for the Ewald sum in the direct and reciprocal space, was set to 10^{-5}. Periodic boundary conditions were employed to mitigate system size effects. Diffusion of water was calculated from mean square displacements over 50 ps using the 'g_msd' tool in GROMACS.